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CONTRACT NUMBER DAMD17-95-C-5004

TITLE: Neuropharmacological Characterization of Botulinum Neurotoxin

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REPORT DATE: April 1997

TYPE OF REPORT: Final

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	April 1997	Final (1 Apr 95 - 31 Mar 97)

4. TITLE AND SUBTITLE
Neuropharmacological Characterization of Botulinum Neurotoxin

5. FUNDING NUMBERS
DAMD17-95-C-5004

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, MD 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200*

The binding of iodinated botulinum toxin to nerve membranes was studied by using rat and mouse preparations. The toxin was examined both in the single-chain and in the proteolytically processed dichain form, and binding sites both in the spinal cord and in various brain regions were assayed. Rat and mouse brains possessed specific binding sites for botulinum toxin. The binding of botulinum toxin to rat brain membranes was not antagonized by a polyclonal antibody against the cytosolic domain of synaptotagmin 1 or by a monoclonal antibody directed against the luminal domain of synaptotagmin 1. In addition, these antibodies did not protect the mouse phrenic nerve-hemidiaphragm from toxin-induced neuromuscular blockade. Extraction of whole-brain mRNA and injection into *Xenopus* oocytes led to expression of binding sites for botulinum toxin. Extraction and injection of cerebellar mRNA led to expression of a higher density of binding sites. The number of binding sites was not diminished when oocytes were pretreated with antibodies against the cytosolic and luminal domains of synaptotagmin 1.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Botulinum toxin, binding sites, oocyte expression system	42
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18

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TABLE OF CONTENTS

INTRODUCTION

1. Background - Mechanism of Toxin Action

Botulinum neurotoxin acts selectively on peripheral cholinergic nerve endings to block the release of acetylcholine. Motor nerve endings are more sensitive than preganglionic or postganglionic nerve endings of the autonomic nervous system, and thus the major outcome of toxin action is blockade of neuromuscular transmission. In the extreme, toxin-induced blockade can produce complete flaccid paralysis.

A general model has emerged to explain the actions of botulinum neurotoxin on sensitive nerve endings (Simpson, 1986; Simpson, 1989). As originally envisioned, the model proposed three sequential steps [i.e., binding, internalization, and intracellular action; (Simpson, 1980)]. However, it is useful to divide the internalization step into two separate events (e.g., penetration of the plasma membrane and penetration of the endosome membrane). This is particularly helpful in the context of efforts to develop pharmacological antagonists of toxin action. Therefore, botulinum neurotoxin action will be described as a series of four events.

Binding. Botulinum toxin binds selectively to motor nerves, and even more precisely to those portions of the presynaptic membrane that are juxtaposed to endplate regions (Black and Dolly, 1986a; Black and Dolly, 1986b). The toxin does not bind to muscle, to myelinated or demyelinated segments of axon, or to tissues remote from the nerve terminal.

The receptor for botulinum neurotoxin has not been isolated and characterized, but there are at least three features of the receptor that have been established. First, the binding of botulinum neurotoxin to the plasma membrane does not alter neuromuscular transmission (Burgen et al., 1949; Simpson, 1980). Thus, it is unlikely that the exposed portion of the receptor plays a critical

role in junctional transmission. Second, there is strong evidence that more than one receptor is implicated in neurotoxin action. Botulinum neurotoxin exists in seven serotypes designated A, B, C, D, E, F and G. These several serotypes do not share a common receptor, and thus there must be multiple receptors that mediate toxin binding (Habermann and Dreyer, 1986; Middlebrook, 1989). Third, there is highly suggestive evidence that a sialic acid-containing molecule plays a role in toxin binding (Simpson and Rapport, 1971a; Simpson and Rapport, 1971b; Bakry et al., 1991a). One possible explanation is that a ganglioside acts cooperatively with another molecule such as a protein to create a binding site (Montecucco, 1986). Another possibility is that the receptor is a sialoglycoprotein (Schiavo et al., 1991).

Endocytosis. Botulinum neurotoxin crosses the plasma membrane by the process of receptor-mediated endocytosis, and this process is blocked by low temperature and by drugs that poison cellular metabolism (Black and Dolly, 1986a; Black and Dolly, 1986b). The fact that botulinum neurotoxin acts locally at the nerve terminal suggests that it is internalized by a population of endosomes that remains in the nerve ending (viz., endosomes that meld with lysosomes) rather than a population that undergoes retrograde axonal transport to the cell body (viz., endosomes that transport tetanus toxin and various virus particles)

Translocation. Botulinum neurotoxin must escape the endosomal lumen and reach the cytosol to exert its poisoning effects. There is some agreement about initiation of this event, but there is uncertainty about how it is completed. Most investigators agree that translocation is an acid-dependent event (for review, see Simpson, 1993a). As the proton pump in the endosome membrane progressively lowers intraluminal pH, the toxin molecule undergoes a conformational change. The most important aspect of this conformational change is exposure of an occult hydrophobic domain. This newly exposed hydrophobic region inserts into the endosome

membrane, thus triggering translocation, but it is unclear how translocation is actually achieved. Various possibilities are: *i.)* a portion of the molecule may serve as a tunnel protein to accommodate passage of the poisoning domain, *ii.)* a portion of the molecule may serve as a leader sequence to achieve translocation, *iii*) several hydrophobic domains may act in concert to achieve a form of bulk transfer, or *iv.)* the molecule could lyse the endosome membrane. There is as yet no compelling evidence in favor of any particular model

Intracellular poisoning. In the recent past there has been a surge of published work dealing with the intracellular effects of clostridial neurotoxins. Three hypotheses have emerged as proposed explanations for toxin action: *i.)* inhibition of intracellular fusogens, *ii.)* stimulation of transglutaminase and intracellular cross-linking, and *iii.)* proteolytic cleavage of peptides that mediate membrane fusion.

Investigators in the Department of the Army have done work that relates botulinum toxin to phospholipase and the fusogenic properties of arachidonic acid (Ray et al., 1993). These investigators conducted experiments with an array of pharmacological agents that were added to the extracellular medium bathing cells, and their results led them to conclude that "botulinum toxin blocks acetylcholine release by lowering the intraterminal arachidonic acid level".

Although some of the findings of Ray et al. are interesting, there is reason to be cautious. Two issues evoke special concern. First, no data were presented that actually prove that intracellular phospholipase or arachidonic acid control exocytosis. Second, it is difficult to see how extracellular phospholipase and/or arachidonic acid can mimic the putative intracellular actions of these agents in stimulating release of acetylcholine. Exocytosis of transmitter is a regulated event, not a constitutive one. Virtually all research on neurotransmitter release envisions the process as one in which there is a rapid sequence of signal-mediated events. The

slow and constant flux of an extracellular substance into the intracellular milieu does not appear to fit well with a process that is mediated by rapid signal transduction.

In addition to conceptual difficulties with the phospholipase/arachidonic acid model, there is an empirical matter. The natural target for botulinum toxin action is the cholinergic neuromuscular junction. Therefore, a series of studies were conducted on the murine phrenic nerve-hemidiaphragm preparation that are identical to those described by Ray et al. (1993) on PC12 cells. Whereas Ray et al. (1993) found that phospholipase A2, arachidonic acid and mellitin produced complete reversal of botulinum neurotoxin action on PC12 cells, the applicant and his colleagues have found that none of these agents produced significant reversal of toxin action on the neuromuscular junction (Coffield et al., 1994).

In a completely different vein, Facchiano, Luini and their colleagues (Facchiano and Luini, 1992; Facchiano et al., 1993) found that tetanus toxin is a substrate for transglutaminase, but more importantly, the toxin can stimulate transglutaminase to act on other substrates. In their more recent paper, Facchiano et al. (1993) found that tetanus toxin induces transglutaminase to produce cross-linking of synapsin. They speculated that tetanus toxin might stimulate cross-linking by proteolytically converting transglutaminase from an inactive precursor to an active product.

If tetanus toxin did indeed stimulate transglutaminase to cross-link synapsin, that might modify exocytosis. However, there are reasons to be cautious about this work. Some concerns are: 1.) There is no compelling evidence that transglutaminase is involved in exocytosis; in fact, the published work cited by Facchiano and Luini is inconclusive and contradictory. 2.) The concentrations of toxin that produce stimulation were rather high (10-8 M and higher). 3.) There was the curious finding that stimulation was maximal when the stoichiometry of toxin to

transglutaminase was 1:1; there was little or no stimulation at ratios of 0.1 to 1.0, and stimulation declined when the ratio was greater than 1.0 to 1.0. This bears no relationship to in vivo or in vitro dose-response studies on tetanus toxin. 4.) There is no evidence that tetanus toxin stimulates transglutaminase in intact cells, nor is there evidence that the toxin cleaves transglutaminase.

The applicant and his colleagues have found additional reasons to be cautious about the transglutaminase hypothesis. To begin with, purified tetanus toxin produced no stimulation of transglutaminase at concentrations relevant to toxicity (i.e., subnanomolar). Furthermore, tetanus toxin: 1.) produced blockade of exocytosis when the ambient levels of calcium were too low to support the calcium-dependent actions of transglutaminase, 2.) acted in the presence of transglutaminase inhibitors, and 3.) did not produce proteolytic cleavage that activated transglutaminase. These findings suggest that it would be premature to implicate transglutaminase in the mechanism of tetanus toxin action.

The third line of investigation, dealing with proteolytic activity of the toxin, is the one that holds greatest promise. This line of investigation actually began in 1986, when Eisel et al. (1986) and Fairweather and Lyness (1986) reported the complete primary sequence of tetanus toxin. Shortly thereafter, Binz et al. (1990) reported the complete sequence for botulinum neurotoxin type A and drew attention to a crucial structural determinant. This toxin - as well as all other clostridial neurotoxins that have been sequenced - possesses the motif HExxH.

The significance of the motif became apparent when Wright et al. (1992) and Schiavo et al. (1992b) noted that it was characteristic of a particular family of zinc-dependent metalloendopeptidases. Schiavo et al. (1992a) then made the discovery that tetanus toxin and botulinum neurotoxin type B were indeed metalloendopeptidases, and synaptobrevin was a

substrate. Link et al. (1992) separately and independently discovered that synaptobrevin was a target for tetanus toxin action. More recently, Montecucco and his colleagues and Niemann, Jahn and their associates have shown that six of the seven serotypes of botulinum neurotoxin are zinc-dependent proteases that cleave synaptobrevin or related proteins that govern vesicle fusion and exocytosis (Huttner, 1993). In addition, the applicant's laboratory has found that chelation of zinc strongly inhibits the neuromuscular blocking properties of all serotypes of botulinum toxin (Simpson et al., 1993a).

The data that are now available favor the idea that clostridial neurotoxins are proteases that block exocytosis by modifying peptides essential for transmitter release. Indeed, the work by Montecucco and his colleagues and by Niemann and Jahn and their associates is the most important that has ever been done on clostridial neurotoxins. Among other things, their work sets the stage for these toxins to enter the age of molecular medicine.

2. Background - Pharmacological Implications

One of the most important implications to stem from work on mechanism of toxin action is that it gives direction to work aimed at finding pharmacological antagonists. Indeed, there is already a rapidly growing literature on agents that delay or block the actions of botulinum neurotoxin and tetanus toxin. This literature indicates that pharmacological antagonists should be divided into two classes: differential (or selective) and universal (Simpson, 1993b). Differential antagonists are those that alter the paralytic effects of some clostridial neurotoxins but not others. The aminopyridines are examples of differential antagonists. Universal antagonists are drugs that significantly alter the biological activity of all serotypes of botulinum neurotoxin as well as tetanus toxin. These drugs are the ones that are most likely to have therapeutic utility.

There are now several groups of drugs that are recognized as universal antagonists. Each of these has a rational origin that is linked to the proposed mechanism of toxin action. As noted above, toxin action begins with binding to receptors on the cell surface. These receptors have not been isolated and characterized, but nevertheless there is evidence to suggest that they have exposed sialic acid moieties. This led to the testing of lectins with affinity for sialic acid as potential toxin antagonists. The work was successful, resulting in the discovery that two such lectins (Limax flavus, Triticum vulgaris) are universal antagonists of toxin binding (Bakry et al., 1991a).

The next step in toxin action is receptor-mediated endocytosis. There are procedures (e.g., lowering temperature) and drugs (e.g., metabolic poisons) that block endocytosis and delay toxicity (Black and Dolly, 1986a; Black and Dolly, 1986b), but these procedures and drugs have two serious limitations. Firstly, they are not selective for endocytosis; they exert effects on aspects of cell function other than endocytosis. Secondly, it is implausible that any of them could be used in a therapeutic context. Thus, it must be acknowledged that there is as yet no selective technique for blocking endocytosis of toxin that could be used in a clinical setting.

The situation with penetration of the plasma membrane is strikingly different from that for penetration of the endosome membrane. There are now two pharmacological approaches for achieving protection. As described above, translocation is an acid-dependent event. This suggests that drugs that block acidification of the endosome or drugs that neutralize endosomal contents should afford protection. Examples of both have been described. Several years ago, it was reported that drugs that neutralize endosomes and lysosomes are universal toxin antagonists. This includes the drugs ammonium chloride, methylamine hydrochloride and chloroquine (Simpson, 1982; Simpson, 1983). Interestingly, the latter agent is already used in medicine (anti-

malarial), and its clinical effects may be related to neutralization of vacuoles. More recently, bafilomycin A1 has been shown to be a universal antagonist (Simpson et al., 1993b). This drug acts selectively on endosomal and other vacuolar membranes to inhibit the ATPase that supports the proton pump. As a result, it blocks acidification of endosomes. Bafilomycin A1 is a universal antagonist of clostridial neurotoxins (Simpson et al., 1993b).

The final step in toxin action is blockade of exocytosis, and this is likely due to expression of zinc-dependent metalloprotease action. This has led to the testing of zinc chelators as toxin antagonists, and the approach has worked well. Selective zinc chelators, such as tetrakis(2-pyridylmethyl)ethylenediamine, antagonize the neuromuscular blocking properties of all clostridial neurotoxins (Schiavo et al., 1992a; Simpson et al., 1993a). This group of antagonists is especially noteworthy, because their actions can be manipulated merely by washing tissues or by adding zinc.

3. Experimental Hypothesis

The development of drugs to delay or prevent botulism is linked to an understanding of mechanism of toxin action. Each advance in the level of understanding creates the potential opportunity for identification of more effective drugs. In fact, a number of pharmacological antagonists have already been described, as discussed above, but it is doubtful that any of them would gain widespread clinical acceptance. Therefore, it is imperative to further clarify toxin action, with the expectation that an authentically efficacious drug will emerge.

Of the several steps in toxin action that one might wish to block, the most appropriate is the extracellular binding step. Antagonism at any of the subsequent steps would likely result in a delay until onset of poisoning, not actual blockade of poisoning. By contrast, inhibition of binding could prevent toxin from reaching the cell interior and producing proteolysis of substrates.

There does exist a literature on toxin binding to receptors, but this literature is very modest in its content and to date has revealed relatively little about the structure and function of toxin receptors. It is not much of an exaggeration to say that almost the only thing known about receptors for serotypes A, B, and E -- the serotypes most often implicated in human poisoning -- is that they are not identical. Individual serotypes appear to have their own unique receptors.

The rate at which knowledge about receptors is accumulating would be substantially increased if there were a satisfactory model system that facilitated receptor research. An ideal model system would have at least four qualities:

- allow expression of receptors for all serotypes of botulinum neurotoxin
- allow expression of either laboratory animal or human receptors
- have a "survivability" and therefore a potential for longterm studies that exceeds that of excised neuromuscular junctions or synaptosomes
- facilitate isolation and characterization of receptors, and thus facilitate testing of potential therapeutic agents that act by antagonizing toxin binding.

There is now substantial evidence arising from several areas of research to show that the Xenopus oocyte system possesses all of these favorable characteristics. Therefore, the work conducted under the aegis of this contract has been to exploit this expression system to achieve the following three specific goals:

i.) Develop the Xenopus oocyte system as a model for expression of, and subsequent investigation of, botulinum neurotoxin receptors.

ii.) Use the oocyte system to express and characterize human receptors for clostridial neurotoxins.

iii.) Seek to isolate receptors for individual serotypes of botulinum neurotoxin, and in the process attempt to identify pharmacological agents that will antagonize toxin binding to receptors.

BODY

1. Experimental Techniques

There are three major techniques that were used during the proposed studies: *i.)* injection of Xenopus oocytes with mRNA to evoke expression of toxin receptors, *ii.)* iodination of botulinum neurotoxin to conduct ligand binding studies on oocytes, and *iii.)* isolation of mouse phrenic nerve-hemidiaphragm preparations to do toxicity experiments.

Oocyte expression system. The bulk of the proposed work was done on oocytes obtained from the African clawed toad Xenopus laevis. This is the most widely used expression system for exogenous translation products (cf. Gurdon and Wickens, 1983; Dascal, 1987), and it is the one currently used by the group at Jefferson. Xenopus are anesthetized with a solution containing 0.15% tricaine, and surgical techniques are used to remove one or more ovarian lobes. The eggs are defolliculated by incubation in calcium-free solution containing collagenase (2 mg/ml), and approximately 24 hours later healthy eggs are microinjected with RNA. The site of injection is the border between the animal and vegetal membranes. Electrophysiologic, ligand-binding or other types of experiments are carried out two to four days later.

Ligand binding studies. Individual serotypes of botulinum neurotoxin were labeled as previously described (Bakry et al., 1991a,b). Labeled toxin was then used on membrane preparations of oocytes to determine number of binding sites, affinity of binding, and in certain cases antagonism of binding.

Membrane preparations were generated by homogenizing oocytes in iced Tris-HCl buffer (50 mM, pH 7.4). Homogenate was centrifuged for 10 minutes at 1000 x g to remove debris, and the resulting supernatant was recentrifuged and used in binding assays.

The binding of toxins to membrane preparations was measured using a centrifugation assay. Labeled toxin was mixed with a predetermined amount of membrane protein in 1.0 ml of pH 7.4 buffer containing 50 mM Tris-HCl, 100 mM NaCl and 1 mg/ml of BSA. The binding reaction was done at 20° C for the amount of time that is empirically determined to be necessary to reach equilibrium. The reaction was terminated by centrifugation (15,000 x g, 2 min), after which the pellet was washed and recentrifuged in fresh buffer. The amount of iodinated ligand in the final pellet was quantified and expressed per concentration of protein or per egg. The data were corrected for nonspecific binding.

The results were evaluated by using the equilibrium binding data analysis program (McPherson, 1982). When more than one binding site per ligand is apparent, the SCAFIT program was used for nonlinear regression analysis (Munson and Rodbard, 1980).

The general strategy for the proposed work was as follows. A single toxin, such as botulinum neurotoxin type A, was chosen for study. The labeled toxin was incubated with varying amounts of membrane protein and submitted to a centrifugation assay to determine the midpoint of the linear portion of the binding curve. Preliminary data suggested that this is likely to be in the range of 100 μg protein. The next step was to use a fixed amount of protein and varying amounts of toxin. Data from this experiment were transformed into a Scatchard plot, from which the investigators could deduce the number of binding sites (viz., high affinity vs. low affinity), the Kd for each site and the Bmax for each site. The same data can be transformed to obtain a Hill analysis, indicating any possible negative or positive cooperativity of binding.

The final step in the general approach was to measure the on-rate and off-rate constants. The former was done by using a fixed concentration of labeled toxin and membrane protein, but varying the incubation time. The latter was done by first allowing equilibrium to be attained between a fixed amount of membrane protein and labeled toxin. This preparation was then washed free of unbound toxin and/or incubated with a large molar excess of unlabeled toxin (to prevent iodinated toxin from associating or reassociating with receptors). After various intervals of time, the preparations were submitted to a centrifugation assay to quantify amount of retained label. The ratio of the rate constants was then used to calculate the apparent Kd. Thus, there were two independent measures of the affinity constant: the Kd derived from the Scatchard plot, and the Kd derived from the rate constant experiments and calculated from the ratio of k_{off}/k_{on}.

There is an additional technique that was used to determine affinity constants. A fixed amount of labeled toxin and a fixed amount of membrane protein was mixed with increasing concentrations of unlabeled toxin. By plotting the percent of labeled toxin that was bound versus the concentration of unlabeled toxin in the incubation mixture, one could obtain a graphic representation of the concentration of unlabeled material that produces 50% inhibition of binding of labeled material. The IC50 for unlabeled toxin should be a close approximation of the Kd for homologous, labeled toxin as determined by the adsorption binding technique and the ratio of rate constants.

Neuromuscular preparations. Mouse phrenic nerve-hemidiaphragm preparations were excised and suspended in physiological medium, as previously described (Simpson et al., 1993a,b). Temperature was maintained at 35°C to 36°C. These preparations were used to monitor indirectly-evoked muscle twitch, directly-evoked muscle twitch, spontaneous miniature endplate potentials, resting membrane potentials and action potentials.

During experiments on indirectly-evoked muscle twitch, tissues were suspended in a 25 ml bath. Phrenic nerves were stimulated at a rate of 0.1 Hz, and muscle responses were measured with a strain gauge transducer and physiological recorder. During other experiments, tissues were pinned in a small Petri dish (5 ml) and continuously perfused (1 ml/min) with fresh physiological solution. The phrenic nerve was stimulated (rate, 1 Hz; current pulse, 1 msec) with a suction electrode filled with 3 M KCl, and standard techniques were used to obtain recordings with glass microelectrodes filled with 3 M KCl (tip resistances, 20 to 40 mX).

Endplate activity, nerve activity, and muscle cell physiology were recorded with a high input impedance amplifier. The output from the amplifier was further amplified, filtered at 5 kHz by a low pass filter and digitized through an A/D converter interfaced with a computer. Data acquisition and analyses were done using the Axtape and Pclamp software. Separation of endplate activity from background was achieved with a window discriminator in the software. In general, a minimum of 7-10 different endplates were sampled per experiment, and activity was recorded for a period of 1-2 mins per endplate. The number of spontaneous miniature endplate potentials recorded per endplate was then counted and the frequency per second determined.

Isolation of mRNA. Total RNA was extracted and isolated by the RNAzol B method, essentially as described in the product literature (BIOTEX Laboratories, Inc.; Houston, TX). Brain tissue was homogenized in RNAzol B solution with a Brinkmann Polytron®. The homogenate was mixed with chloroform (10 percent by volume) and vigorously shaken for 15 sec. The suspension was centrifuged at 12,000 x g for 15 min, after which the colorless aqueous phase was collected. Total RNA was precipitated by adding an equal volume of isopropanol (0°C, 30 min). Precipitated total RNA was collected and washed once with 70 percent ethanol. mRNA (poly "A" RNA) was separated from total RNA by the Expresep method, as described in

the product literature (BIOTEX Laboratories, Inc.). Isolated mRNA was dissolved in diethylpyrocarbonate-treated water and stored at -80°C

Antibodies. Antibodies specific against VAMP-1 were raised against a peptide corresponding to the amino-terminal 14 amino acids of VAMP-1 (NH_2-MSAPAQPPAEGTEGC-COOH) including an added carboxy-terminal cysteine.

Antibodies specific against VAMP-2 were raised against a peptide corresponding to the amino-terminal 16 amino acids of VAMP-

Antibodies specific against cellubrevin were raised against a peptide corresponding to the amino-terminal 15 amino acids of cellubrevin (NH_2-MSTGVPSGSSAATGSC-COOH) including an added carboxy-terminal cysteine.

In every case, purity of peptides was assessed using both analytical HPLC and mass spectrometery. Peptides were crosslinked to Imject® maleimide activated BSA or Imject® maleimide activated keyhole limpet hemocyanin (Pierce), according to manufacturers instructions. Rabbits were immunized according to standard protocols by certified technicians in our animal colony. Prebleeds and bleeds were also performed by the same technicians under a clinical veterinarian's supervision. Specificity of antibodies was determined using known standards and comparison with other antibodies raised against these same proteins.

Western Blots. Antibodies used for western blots were generated in rabbits according to the method described above. Antibody dilutions were used at 1:200 to 1:1000 for rabbit polyclonal antibodies, and 1:5000 or higher for monoclonals. Western blots were imaged using ECL reagents (Amersham Life Science), according to manufacturers instructions. Briefly, samples were separated using polacrylamide gel electrophoresis (PAGE). Acrylamide gel percentages were adjusted according to sample requirements (7.5 to 15%). Subsequent to separation,

proteins were transferred to NitroPure or NitroBind nitrocellulose transfer membranes (Micron Separations, Inc.), in Tris-Glycine transfer buffer at 50 volts for 30 to 45 minutes. Blotted membranes were rinsed in distilled water and stained for 1 minute with 0.2% Ponceau S in 1% acetic acid. Following a brief rinse with distilled water, standards and lanes were identified on the stained membranes. Membranes were destained in PBS-Tween or TBS-Tween (Phosphate buffered saline pH 7.5 or Tris buffered saline pH 7.6, with 0.1% Tween 20). Destained membranes were blocked with 5% non-fat powdered milk in PBS- or TBS-Tween for 1 hour at room temperature, or overnight at 3°C. Membranes were washed (3X), then incubated with primary antibodies at the appropriate dilution (see above), in 0.05 to 1.0% milk for 2 to 5 hours at room temperature. Membranes were washed (3X), and incubated with the appropriate secondary antibody (Amersham) at 1:10,000 dilution for 1 to 2 hours at room temperature. After a final series of washes (5X), immunodetected proteins were imaged by reacting with ECL reagent for 1 minute, excess reagent was drained and membranes covered with Saran WrapTM and exposed to film (Hyperfilm-ECL) for the amount of time required to visualize chemiluminescent protein bands. Proteins were identified through comparison with known standards.

2. Experimental Findings

Characterization of ligand. Botulinum toxin type B isolated as previously described (Simpson et al., 1988) was collected from a single peak at the conclusion of a series of chromatographic steps. Polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate confirmed that the isolated toxin was essentially homogeneous and in the single chain form (Figure 1). Proteolytic processing of the toxin with agarose-bound trypsin resulted in almost complete conversion to the dichain form (Figure 1).

The relative potencies of the single chain and dichain species were bioassayed on the mouse phrenic nerve-hemidiaphragm preparation, as previously described (Simpson and DasGupta., 1983). Proteolytic processing of the toxin resulted in an increase in potency of more than an order of magnitude (Figure 2).

Single chain and dichain forms of the toxin were radioiodinated, as described under Methods, to give preparations with high specific activity (range, 600 to 900 Ci/mmol). Labeled material was bioassayed for residual toxicity, as described above, and was found to retain between 70 and 90 percent of the activity of native toxin.

Radiolabeled toxin was submitted to polyacrylamide gel electrophoresis, and individual lanes containing the single chain and dichain forms of the toxin were excised and cut into 2 mm strips. As expected, lanes containing the single chain species had a single peak of radioactivity, and this peak migrated in a manner consistent with a molecular weight of ~150,000 (Figure 1). Lanes containing the dichain species had two major peaks, and the molecular weights were consistent with those of the heavy chain (~95,000 to 100,000) and light chain (~51,000) of botulinum toxin. Interestingly, the fraction of radioactivity associated with the heavy chain (~78%) was greater than that predicted on the basis of molecular weight. This means that radioactivity was enriched in that portion of the toxin molecule thought to mediate binding.

Binding of toxin to nerve membrane preparations. In an initial series of experiments, membrane preparations from rat (brain), mouse (brain) and oocytes (whole cells) were tested for their ability to bind iodinated dichain toxin. Standard conditions for this binding assay were: toxin concentration, 0.5 to 1.0 nM; membrane concentration, 100 μg/assay; reaction volume (100 μl); time, 2 hr; and temperature, 23°C. These conditions were similar to those previously used to

study clostridial toxin binding to brain membrane preparations (Bakry et al., 1991, Bakry et al., 1991).

These preliminary experiments indicated that tissues of rat and mouse origin displayed binding sites for toxin. Kinetic analyses and regional binding data are presented below. In contrast, native oocyte membranes had no specific binding sites for toxin. This suggests that oocytes could be an acceptable system in which to induce expression of binding sites.

Characteristics of toxin binding to rat brain and spinal cord membranes. Botulinum toxin type B binding to rat membrane preparations increased as a function of protein (Figure 3A). An apparent plateau was observed in the range of 20 to 30 μg membrane protein per assay (reaction volume, 100 μl). This result was obtained both with brain and with spinal cord. All subsequent experiments were done at a membrane protein concentration of 15 μg per assay.

The binding of botulinum toxin to membrane preparations increased with time, and a true equilibrium was not reached until 60 to 90 min (Figure 3B). This incubation time was incorporated into subsequent experiments.

Temperature had a significant effect on specific binding. The greatest amount of specific binding was obtained at 23°C. This binding was reduced at 37°C, and it was markedly reduced at 0°C (Figure 3C). All subsequent experiments were done at room temperature (~23°C).

Specific binding of the single chain and dichain species of botulinum toxin type B were compared, using the assay conditions described in the preceding paragraphs. Regardless of the origin of tissues, the two species were indistinguishable in their binding characteristics (not illustrated). All subsequent experiments were done with the single chain molecule.

Various concentrations of iodinated botulinum toxin type B were incubated with rat brain membrane preparations, and the resulting data on specific binding were used to generate a

saturation isotherm and a scatchard plot (Figure 4). Graphic analysis of the data revealed a single class of binding sites with a Kd of 2 nM and a Bmax of 2.6 pmol/mg protein. In companion experiments, various concentrations of unlabeled toxin were used as competitive antagonists of labeled toxin binding (Figure 5). The apparent IC50 as deduced by graphic analysis was 2 nM, which is in accord with the Kd value for radioligand binding (see above).

Unlike homologous toxin, heterologous unlabeled toxin was not an effective antagonist of iodinated botulinum toxin type B binding. At concentrations 10-fold greater than the IC50 for unlabeled serotype B, unlabeled serotypes A and C produced less than 20 percent inhibition of iodinated toxin binding (results not illustrated).

In addition to studies on membrane preparations derived from rat whole brain, work was done on preparations from select regions, including the forebrain, basal forebrain, cerebellum, and spinal cord (Table 1). The Kd values for these four regions ranged from 2.9×10^{-9} M (spinal cord) to 5.5×10^{-9} M (forebrain); the Bmax values ranged from 3.0 pmol/mg (forebrain) to 10.3 pmol/mg (cerebellum). The data indicated that the average binding affinity for these four regions was slightly less than that for whole brain (i.e., higher Kd value; see above), but the average density of binding sites was slightly higher.

Botulinum toxin binding to mouse membrane preparations. A more limited series of studies was done on membrane preparations from different regions of mouse brain. Representative data for one specific region (saturation isotherm and resulting scatchard analysis) showed that mouse basal forebrain had a single class of high affinity binding sites for iodinated botulinum toxin (Figure 6). The Kd value was 4.1 nM and the Bmax value was 8.4 pmol/mg protein, which are reasonably similar to the values for whole rat brain preparations.

When three specific tissues were analyzed (cerebellum, forebrain, basal brain), the resulting Kd values fell within a narrow range (2.9×10^{-9} M to 4.3×10^{-9} M), and this range fell within that observed for rat tissues (Table 1). On the other hand, the resulting Bmax values were more disparate. The difference between the mouse cerebellum (12 pmol/mg protein) and the mouse forebrain (2 pmol/mg protein) was six-fold. The relatively high value obtained in mouse cerebellar membranes was exploited in expression experiments.

Expression of binding sites in oocytes. The binding of botulinum toxin to Xenopus oocytes was examined before or after injection of mRNA (see Methods). A single toxin concentration was used (1×10^{-9} M), and conditions for the binding assay were identical to those used with rat brain tissues (see above).

As described earlier, membranes from native oocytes were devoid of specific binding sites. However, in most cases (>80%), injection of message from whole rat brain led to expression of specific binding sites. The average value obtained for membranes in 9 successful experiments was 30 ± 4 fmol/mg protein.

Earlier experiments on rat and mouse brain demonstrated that cerebellum had the highest density of toxin binding sites. Therefore, mRNA from rat cerebellum or mouse cerebellum was injected into oocytes. Interestingly, the success rate in obtaining binding sites rose to 100% when cerebellar mRNA was injected. The actual values obtained were as follows: rat cerebellum mRNA, 40 ± 5 fmol/mg protein (n=4); mouse cerebellum mRNA, 42 ± 10 fmol/mg protein (n=4).

The effects of synaptotagmin antibodies on botulinum toxin binding and activity. Rat brain membrane preparations were incubated (30 min; 23°C) with antibody against either the cytosolic or lumenal domains of synaptotagmin 1. The antibodies were used at a five-fold excess to that which has been shown to bind to and label epitopes on synaptic vesicles (Mundigl et al., 1995).

Iodinated botulinum toxin type B was then added (1×10^{-9} M), and binding was measured as described above. The results indicated that neither antibody reduced the amount of toxin binding (not illustrated).

In related experiments, phrenic nerve-hemidiaphragm preparations were immersed in medium at 5°C, which virtually abolishes both exocytosis and endocytosis. Antibody against either the cytosolic or lumenal domain of synaptotagmin was added, and incubation was continued for 30 min. Botulinum toxin type B (1×10^{-11} M) was then added, and incubation was continued for an additional 30 min. Tissues were washed and suspended in antibody-free and toxin-free solution at 35°C, phrenic nerves were stimulated, and paralysis times were monitored. The results were: control tissues (n=10), 111±9 min; tissues (n=4) incubated with antibody against the cytosolic domain of synaptotagmin (118±12 min); tissues (n=6) incubated with antibody against the lumenal domain of the peptide (121±13 min). Neither antibody produced statistically significant protection against botulinum toxin.

Binding experiments with botulinum toxin type B were redone with oocytes injected with rat cerebellar mRNA. The amount of toxin binding was compared in control preparations, experimental preparations preincubated with antibody against the cytosolic domain of synaptotagmin, and experimental preparations preincubated with antibody against the lumenal domain of the peptide. Preincubation with each antibody was for 30 min at 23°C. The results demonstrated that the amount of specific binding in the three tissue preparations was the same. There was no evidence for antagonism of toxin binding by either antibody.

CONCLUSIONS

Botulinum toxin acts preferentially on cholinergic nerve endings to block acetylcholine release. The toxin produces this effect by proceeding through a sequence of events, the first of which is association with cell surface receptors. The identity of these receptors and their role in nerve function have not been determined (Middlebrook, 1989). Indeed, it has not even been established whether there are simple receptors. Montecucco (Montecucco, 1986) has advanced the interesting idea that binding might be a biphasic process. There could be an initial interaction with lower affinity binding sites (viz., membrane lipids), and this would promote a secondary interaction with higher affinity sites (viz., protein). The value of this proposal and others will not be known until toxin receptors are actually identified.

A strategy for characterizing receptors. A number of investigators have studied labeled botulinum toxin binding to nerve membrane preparations (for representative studies, see Evans et al., 1986, Kozaki, 1979, Williams et al., 1983, Yokosawa et al., 1991). Because the amount of nerve ending tissue at the neuromuscular junction is limited, almost the entire literature on binding deals with brain membrane preparations [but see the work by Black and Dolly, 1986, Black and Dolly 1986)]. This is in keeping with the fact that botulinum toxin blocks transmitter release from isolated brain synaptosomes.

Earlier studies on botulinum toxin type B binding have shown that whole brain preparations have specific, high affinity binding sites (Evans et al., 1986, Kozaki, 1979). Furthermore, these binding sites are unique or relatively unique to this serotype, because heterologous serotypes do not block binding. These earlier studies have established a framework in which the current work was done.

Botulinum toxin type B was labeled to high specific activity, and its binding was studied in several discrete areas of the rat and mouse central nervous system. An initial examination of iodinated type B toxin binding showed that the single chain and dichain species were virtually indistinguishable. This is in marked contrast to findings with other steps in the paralytic process. To begin with, the single chain species does not appear to have the correct conformation and/or flexibility needed for cellular internalization. In addition, the single chain species expresses little or no enzymatic activity, perhaps because of conformational restraints on the catalytic domain. These limitations explain why the single chain molecule is much less potent than the dichain molecule when tested on phrenic nerve-hemidiaphragm preparations.

The membrane preparations used in the present study did not have intact synaptosomes, and thus there should be little possibility that the process of internalization could be mistaken for binding. However, to minimize any difficulties, the bulk of the work was done with the single chain molecule. This also reduced the prospect that toxin~substrate interactions could complicate matters because, as indicated above, the single chain molecule has little enzymatic activity.

Analysis of toxin binding. All regions of rat and mouse central nervous system that were tested had specific binding sites for botulinum toxin type B. In general, the Kd values for the different regions of rat and mouse brain were comparable.

There is one important point that should be made in relation to the binding data. Previous work has shown that botulinum toxin type B is orders of magnitude more potent in blocking mouse than blocking rat neuromuscular transmission (Burgen et al., 1949). To the extent that binding to brain membranes reflects binding to motor nerve membranes, the data suggest that resistance of the rat is not due to an absence of specific receptors (and see Evans et al., 1986). This conclusion meshes well with another line of recent research. Botulinum toxin type B blocks

transmission by cleaving synaptobrevin (also known as VAMP, or vesicle-associated membrane protein) at Gln76-Phe77. Mouse synaptobrevin has this bond, but rat synaptobrevin has a substitution at this site that creates a Val76-Phe77 bond (Patarnello et al., 1993). In all likelihood, resistance of the rat to serotype B is due to an absence of a susceptible cleavage site in the substrate and not to absence of specific receptors.

In contrast to brain and spinal cord, native Xenopus oocytes were devoid of specific binding sites for botulinum toxin type B. However, injection of oocytes with mRNA of whole rat brain origin led to expression of binding sites, and injection of mRNA of either rat cerebellum or mouse cerebellum origin led to expression of even more binding sites. These results are encouraging, not only because they demonstrate that binding sites can be induced in oocytes, but also because they demonstrate that the relative density of binding sites in the expression system mimics the relative density in the donor tissue.

The putative role of synaptotagmin. In the recent past, Nishiki (Nishiki et al., 1994, Nishiki et al., 1993) have published work suggesting that synaptotagmin, perhaps in association with another molecule, may act as a receptor for serotype B. Synaptotagmin is enriched in the membranes of synaptic vesicles. During the process of transmitter release, synaptic vesicles fuse and then meld with the plasma membrane. This means that for a finite period of time the lumenal domain of synaptotagmin is exposed to the cell surface. It is at this point that, hypothetically, synaptotagmin could serve as a whole or partial receptor for the toxin. Therefore, botulinum toxin binding and biological activity were studied in the absence and presence of antibodies against synaptotagmin.

Experiments in which antibodies against the lumenal and cytosolic domains of synaptotagmin 1 were tested as antagonists of toxin binding to brain membrane preparations did not reveal any

protection. The antibodies--like the toxin--are large molecules (>150,000 daltons), which means that antibodies could block toxin binding either by attaching to the toxin binding site (viz., true competition) or by attaching to an adjacent site (viz., steric hindrance). The finding that there was a complete absence of protection suggests that synaptotagmin 1 is not acting as a sole receptor.

The binding studies on brain membrane preparations were supplemented by experiments on oocytes and on phrenic nerve-hemidiaphragm preparations. Native oocytes did not express specific receptors for toxin, but injection of rat or mouse cerebellar mRNA did lead to expression of binding sites. In keeping with the results on brain membranes, antibodies against the two free domains of synaptotagmin 1 did not diminish toxin binding to oocyte membranes. Analogous results were obtained in functional studies with a neuromuscular preparation. Antibodies against synaptotagmin 1 did not alter the amount of time necessary for toxin-induced blockade of transmission in the phrenic nerve-hemidiaphragm.

The neuromuscular junction studies are especially relevant to issues that pertain to toxin binding. As discussed above, it is presumably the lumenal domain of synaptotagmin that would serve as a binding site, and this portion of the molecule would be exposed when exocytotic vesicles meld with the plasma membrane. The number of these binding sites would be low under conditions in which there is little or no exocytosis (e.g., low temperature), but the relative number and rate of turnover would increase when there is substantial exocytosis (e.g., rapid nerve stimulation). Indeed, recent work has demonstrated exactly this outcome. De Camilli and his colleagues (Mundigl et al., 1995) have shown that an iodinated antibody against the lumenal domain of synaptotagmin 1 can be used to measure exocytosis. This work has shown that binding is reduced at low temperature, but binding of the antibody increases at physiological temperature or with nerve depolarization.

Experiments in the present study involved the same antibody as that developed to measure exocytotic activity. The antibody was added to the neuromuscular junction at 4°C for 30 min, which is more than adequate time for equilibrium to be obtained [ca. 3 to 4 min; (Mundigl et al., 1995)]. Botulinum toxin was then added for 30 min, after which tissues were washed and suspended in physiological solution at 35°C. The results demonstrated that prior treatment of tissues with antibodies against synaptotagmin 1 did not afford any protection against botulinum toxin type B.

The combined results raise questions about the role of synaptotagmin 1 as a sole receptor for botulinum toxin. However, they do not necessarily challenge the premise that the peptide could be one part of a dual receptor. The absence of antibody protection in binding experiments could be explained on the basis that toxin was associating with the non-synaptotagmin portion of the dual receptor. The absence of protection in functional experiments could be explained on the same basis, with the assumption that the toxin becomes associated with newly exposed synaptotagmin when nerve stimulation begins.

Another and perhaps more important point is that synaptotagmin 1 is only the first in what is now a growing family of synaptotagmins. It is possible that another of the several forms of synaptotagmin is the full or partial receptor for serotype B. Indeed, this concept is strengthened by a report that appeared while the current work was in progress. Nishiki, (Nishiki et al., 1996) have found that botulinum toxin has a greater affinity for synaptotagmin 2 than synaptotagmin 1. This finding suggests that serotype B should be examined for its ability to bind to all members of the synaptotagmin family. The resulting data on affinity constants could be used as a basis for deciding which synaptotagmin to express in oocytes in an effort to reconstitute botulinum toxin binding sites.

FIGURES AND TABLES

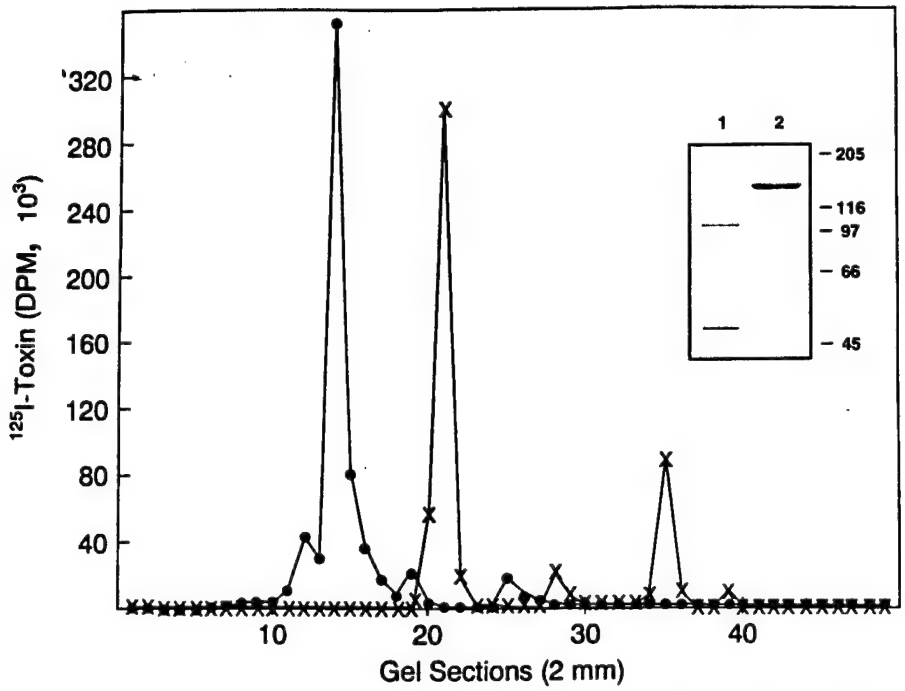

FIG. 1. Botulinum neurotoxin type B was isolated in the single-chain form and then converted by proteolysis to the more active dichain form (see text for details). The inset panel illustrates the migration of the activated (lane 1) and nonactivated (lane 2) toxin during polyacrylamide gel electrophoresis in the presence of reducing agent (numbers to the right of the gel are molecular weights [in thousands]). The same amount of protein (1.3 μg) was added to both lanes. The single-chain and dichain forms of the toxin were subjected to two additional procedures. The single-chain and dichain molecules were radiolabeled with Bolton-Hunter reagent as described in the text and then run on reducing gels as indicated above. The gels were cut into 2-mm-wide sections, and the amount of radioactivity was quantified. The results demonstrated that radioactivity of the single-chain preparation (●) migrated in accordance with the known molecular weight of the holotoxin (ca. 150,000) and that the radioactivity of the dichain preparation (×) migrated in accordance with the molecular weights of the heavy chain and light chain (ca. 100,000 and 51,000). The two forms of botulinum neurotoxin type B were also bioassayed for activity. The results are illustrated in Fig. 2.

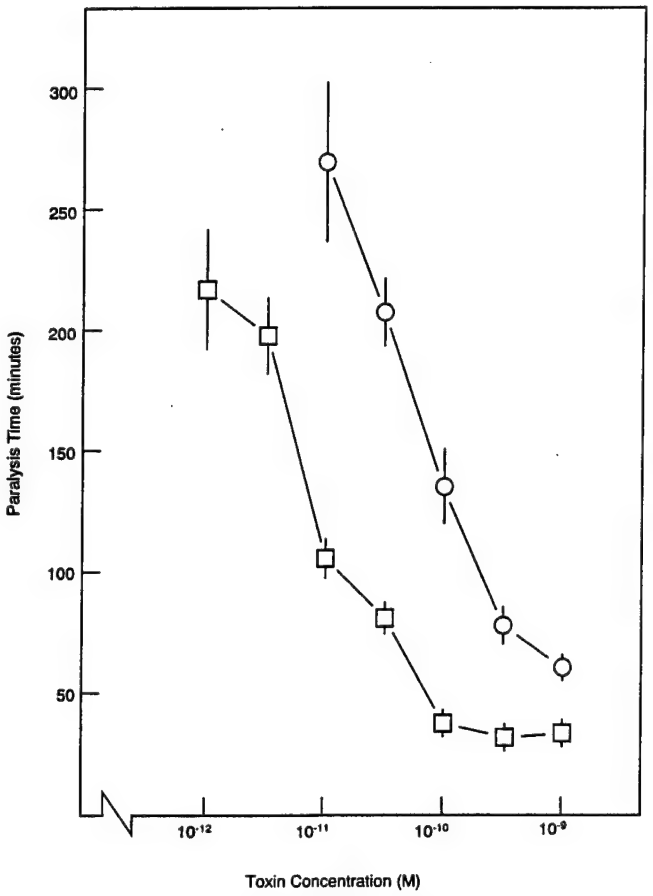

FIG. 2. The single-chain (○) and dichain (□) forms of botulinum neurotoxin were assayed for activity on the mouse phrenic nerve-hemidiaphragm preparation. Nerves were stimulated at a rate of 1.0 Hz. Paralysis time was defined as the amount of time necessary for toxin to produce a 90% reduction in nerve stimulus-evoked muscle responses. Each data point represents the mean ± standard error of the mean (error bar) of four or more nerve preparations. Note that proteolysis of the toxin was associated with a shift in the concentration-response curve of more than an order of magnitude.

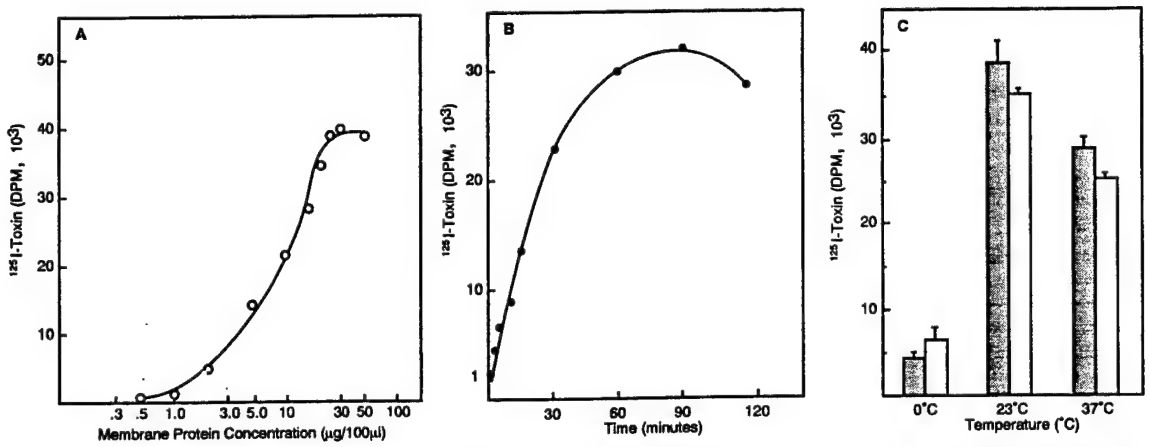

FIG. 3. A variety of conditions were examined in an effort to optimize the binding of botulinum neurotoxin to rat brain membrane preparations. (A) Various concentrations of membrane preparations from whole rat brain were incubated with iodinated botulinum neurotoxin (5×10^{-10} M; single chain). Incubation was for 120 min at room temperature (23°C). An apparent plateau in binding was observed at 20 to 30 µg of protein per assay. Each data point is the mean value obtained from three experiments, with each experiment done in triplicate. (B) Brain membrane preparations (15 µg of protein) were incubated with a fixed concentration of toxin (5×10^{-10} M; single chain; 23°C) for various lengths of time. An apparent equilibrium was reached in 60 to 90 min. Each data point is the average value obtained from three experiments, each of which was done in triplicate. (C) Botulinum neurotoxin (5×10^{-10} M) was incubated with membrane preparations (15 µg of protein: 120 min) at one of three temperatures (0, 23, or 37°C). The toxin was examined in both the single-chain (shaded bars) and dichain (open bars) forms. The values are the means of two experiments, each done in triplicate. For all three panels of the figure, the results are expressed as specific binding (i.e., corrected for nonspecific binding, which was less than 10%, in the presence of a 50-fold excess of unlabeled toxin).

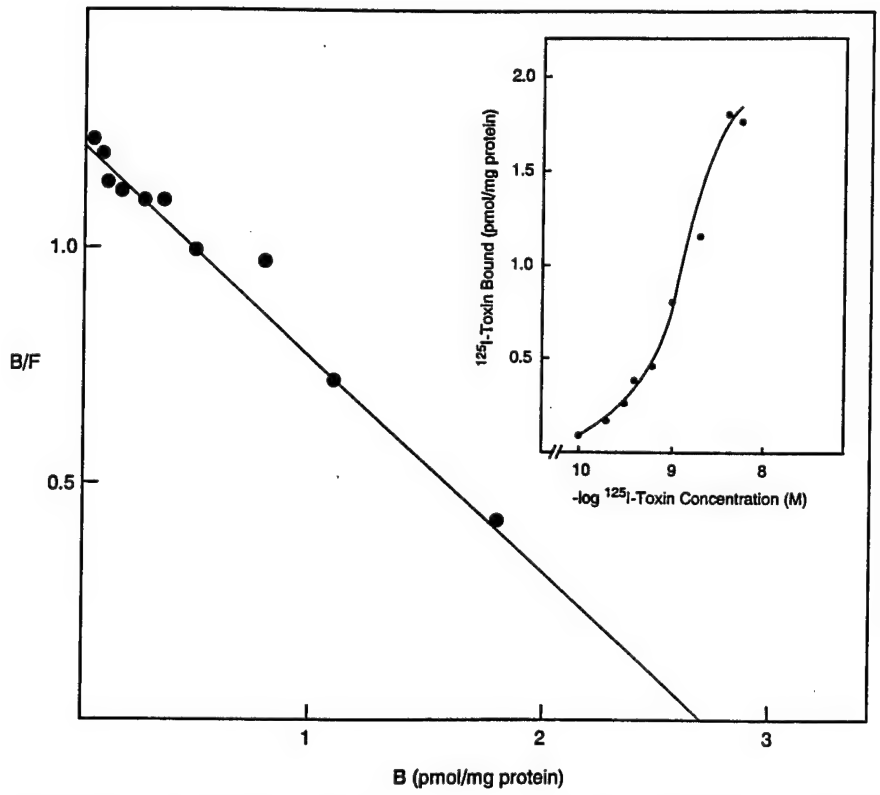

FIG. 4. Various concentrations of iodinated toxin were incubated with a fixed amount of membrane preparations of rat whole-brain origin (inset). Optimized conditions for specific binding were used, as described in the text and in the legend to Fig. 3. The resulting data were transformed into a Scatchard plot. The figure illustrates the data from one representative experiment. A total of five experiments were done, with each individual experiment done in triplicate. The average K_d for all experiments was 2 nM, and the average B_{max} was 2.6 pmol/mg of protein.

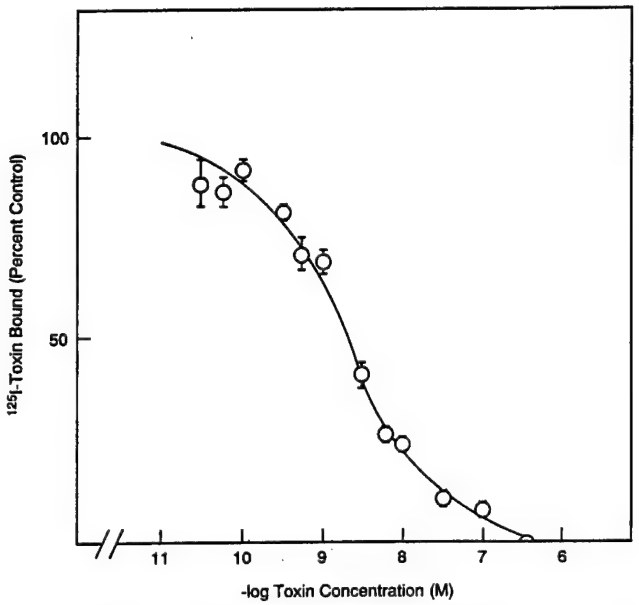

FIG. 5. Various concentrations of unlabeled type B toxin were tested for competitive antagonism of the specific binding of labeled type B toxin (5×10^{-10} M). In these experiments, unlabeled toxin was added approximately 2 min before labeled toxin. Optimized conditions for binding were used, as described in the text. Each data point reflects the mean ± standard error of the mean (error bars) of three separate experiments, each done in triplicate. The concentration of unlabeled toxin that produced 50% inhibition of binding of labeled toxin was ca. 2×10^{-9} M.

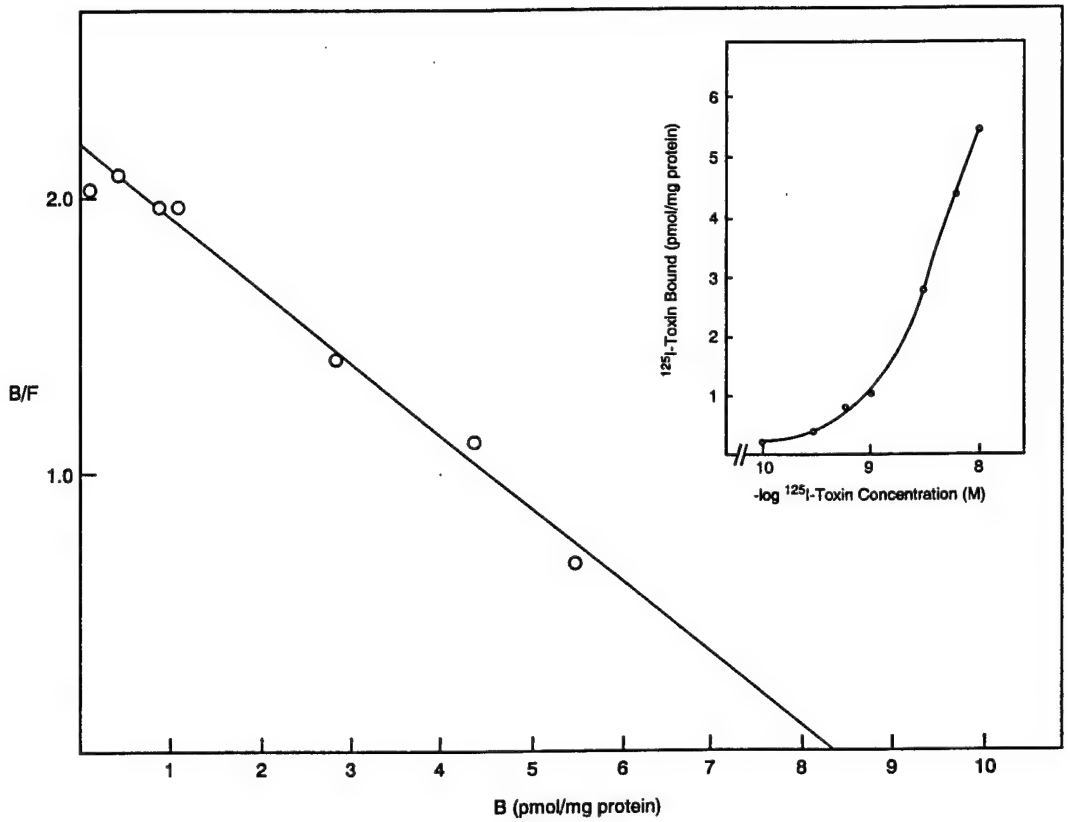

FIG. 6. Various concentrations of iodinated type B toxin were incubated with membrane preparations of mouse brain origin (inset). Optimized conditions for specific binding were used, as previously described. The resulting data were transformed into a Scatchard plot. The figure illustrates one of three separate experiments, each of which was done in triplicate. The average K_d for all experiments was 4.1 nM, and the average B_{max} was 8.4 pmol/mg of protein.

TABLE 1. Specific binding of ^{125}I-botulinum neurotoxin type B to preparations from different areas of rat and mouse central nervous systems

Animal and tissue	K_d (nM)[a]	B_{max} (pmol/mg of protein)
Rat		
Forebrain	5.5 ± 1.0	3.0 ± 0.3
Basal forebrain	4.8 ± 1.1	7.7 ± 0.8
Cerebellum	3.5 ± 0.4	10.3 ± 0.6
Spinal cord	2.9 ± 0.3	8.0 ± 1.0
Average	4.2 ± 0.7	7.3 ± 0.7
Mouse		
Forebrain	2.9 ± 0.9	2.0 ± 0.6
Basal forebrain	4.1 ± 1.2	8.4 ± 1.5
Cerebellum	4.3 ± 0.6	12.0 ± 3.5
Average	3.7 ± 0.9	7.5 ± 1.9

[a] The binding of iodinated toxin to membrane preparations was examined under the following conditions: membrane protein concentration, 15 μg per assay; incubation time, 60 to 90 min; and incubation temperature, 23°C. Specific binding was quantified by subtracting nonspecific binding (e.g., iodinated toxin in the presence of a 50-fold molar excess of unlabeled toxin) from total binding (e.g., iodinated toxin alone). Values are expressed as means ± standard errors of the means. For each group, n is ≥3.

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